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(54) Title: EXPRESSION SYSTEM

(57) Abstract: An immunogenic reagent which produces an immune response which is protective against *Bacillus anthracis*, said reagent comprising one or more polypeptides which together represent up to three domains of the full length Protective Antigen (PA) of *B. anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof. The polypeptides of the immunogenic reagent as well as full length PA are produced by expression from *E. coli*. High yields of polypeptide are obtained using this method. Cells, vectors and nucleic acids used in the method are also described and claimed.



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## Expression System

The present invention relates to polypeptides which produce an immune response which is protective against infection by  
5 *Bacillus anthracis*, to methods of producing these, to recombinant *Escherischia coli* cells, useful in the methods, and to nucleic acids and transformation vectors used.

Present systems for expressing PA for vaccine systems use  
10 protease deficient *Bacillus subtilis* as the expression host. Although such systems are acceptable in terms of product quantity and purity, there are significant drawbacks. Firstly, regulatory authorities are generally unfamiliar with this host, and licensing decisions may be delayed as a result. More  
15 importantly, the currently used strains of *Bacillus subtilis* produce thermostable spores which require the use of a dedicated production plant.

WO00/02522 describes in particular VEE virus replicons which  
20 express PA or certain immunogenic fragments.

*E. coli* is well known as an expression system for a range of human vaccines. While the ability to readily ferment *E. coli* to very high cellular densities makes this bacterium an ideal host  
25 for the expression of many proteins, previous attempts to express and purify recombinant PA from *E. coli* cytosol have been hindered by low protein yields and proteolytic degradation (Singh et al., J. Biol. Chem. (1989) 264; 11099-11102, Vodkin et al., Cell (1993) 34; 693-697 and Sharma et al., Protein Expr.  
30 purif. (1996), 7, 33-38).

A strategy for overexpressing PA as a stable, soluble protein in the *E. coli* cytosol has been described recently (Willhite et al., Protein and Peptide Letters, (1998), 5; 273-278). The  
35 strategy adopted is one of adding an affinity tag sequence to the N terminus of PA, which allows a simple purification system.

A problem with this system is that it requires a further downstream processing step in order to remove the tag before the PA can be used.

- 5 Codon optimisation is a technique which is now well known and used in the design of synthetic genes. There is a degree of redundancy in the genetic code, in so far as most amino acids are coded for by more than one codon sequence. Different organisms utilise one or other of these different codons  
10 preferentially. By optimising codons, it is generally expected that expression levels of the particular protein will be enhanced.

This is generally desirable, except where, as in the case of PA,  
15 higher expression levels will result in proteolytic degradation and/or cell toxicity. In such cases, elevating expression levels might be counter-productive and result in significant cell toxicity.

- 20 Surprisingly however, the applicants have found that this is not the case in *E. coli* and that in this system, codon optimisation results in expression of unexpectedly high levels of recombinant PA, irrespective of the presence or absence of proteolytic enzymes within the strain.

25

Furthermore, it would appear that expression of a protective domain of PA does not inhibit expression in *E. coli*.

- The crystal structure of native PA has been elucidated (Petosa  
30 C., et al. Nature 385: 833-838,1997) and shows that PA consists of four distinct and functionally independent domains: domain 1, divided into 1a, 1-167 amino acids and 1b, 168-258 amino acids; domain 2, 259-487 amino acids; domain 3, 488-595 amino acids and domain 4, 596-735 amino acids.

35

The applicants have identified that certain domains appear to produce surprisingly good protective effects when used in isolation, in fusion proteins or in combination with each other.

- 5 According to the present invention there is provided an immunogenic reagent which produces an immune response which is protective against *Bacillus anthracis*, said reagent comprising one or more polypeptides which together represent up to three domains of the full length Protective Antigen (PA) of *B.*  
10 *anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof.

Specifically, the reagent will comprise mixtures of polypeptides or fusion peptides wherein individual polypeptides comprise one  
15 of more individual domains of PA.

In particular, the reagent comprises polypeptide(s) comprising domain 1 or domain 4 of PA or a variant thereof, in a form other than full length PA. Where present, domains are suitably  
20 complete, in particular domain 1 is present in its entirety.

The term "polypeptide" used herein includes proteins and peptides.

- 25 As used herein, the expression "variant" refers to sequences of amino acids which differ from the basic sequence in that one or more amino acids within the sequence are deleted or substituted for other amino acids, but which still produce an immune response which is protective against *Bacillus anthracis*. Amino  
30 acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be  
35 possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical,

preferably at least 75% identical, and more preferably at least 90% identical to the PA sequence.

In particular, the identity of a particular variant sequence to the PA sequence may be assessed using the multiple alignment method described by Lipman and Pearson, (Lipman, D.J. & Pearson, W.R. (1985) Rapid and Sensitive Protein Similarity Searches, Science, vol 227, pp1435-1441). The "optimised" percentage score should be calculated with the following parameters for the Lipman-Pearson algorithm: ktup =1, gap penalty =4 and gap penalty length =12. The sequences for which similarity is to be assessed should be used as the "test sequence" which means that the base sequence for the comparison, (SEQ ID NO 1), should be entered first into the algorithm.

Preferably, the reagent of the invention includes a polypeptide which has the sequence of domain 1 and/or domain 4 of wild-type PA.

A particularly preferred embodiment of the invention comprises domain 4 of the PA of *B. anthracis*.

These domains comprise the following sequences shown in the following Table 1.

Domain	Table 1
	Amino acids of full-length PA*
4	596-735
1	1-258

These amino acids numbers refer to the sequence as shown in Welkos et al. Gene 69 (1988) 287-300 and are illustrated hereinafter as SEQ ID NOs 15 (Fig 4) and 3 (Fig 3) respectively.

Domain 1 comprises two regions, designated 1a and 1b. Region 1a comprises amino acids 1-167 whereas region 1b is from amino acid 168-258. It appears that region 1a is important for the

production of a good protective response, and the full domain may be preferred.

In a particularly preferred embodiment, a combination of domains  
5 1 and 4 or protective regions thereof, are used as the immunogenic reagent which gives rise to an immune response protective against *B. anthracis*. This combination, for example as a fusion peptide, may be expressed using the expression system of the invention as outlined hereinafter.

10

When domain 1 is employed, it is suitably fused to domain 2 of the PA sequence, and may preferably be fused to domain 2 and domain 3.

15 Such combinations and their use in prophylaxis or therapy forms a further aspect of the invention.

Suitably the domains described above are part of a fusion protein, preferably with an N-terminal glutathione-s-transferase  
20 protein (GST). The GST not only assists in the purification of the protein, it may also provide an adjuvant effect, possibly as a result of increasing the size.

The polypeptides of the invention are suitably prepared by  
25 conventional methods. For example, they may be synthesised or they may be prepared using recombinant DNA technology. In particular, nucleic acids which encode said domains are included in an expression vector, which is used to transform a host cell. Culture of the host cell followed by isolation of the desired  
30 polypeptide can then be carried out using conventional methods. Nucleic acids, vectors and transformed cells used in these methods form a further aspect of the invention.

Generally speaking, the host cells used will be those that are  
35 conventionally used in the preparation of PA, such as *Bacillus subtilis*.

The applicants have found surprisingly that the domains either in isolation or in combination, maybe successfully expressed in *E. coli* under certain conditions.

5 Thus, the present invention further provides a method for producing an immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising transforming an *E. coli* host with a nucleic acid which encodes either (a) the protective antigen (PA) of *Bacillus*  
 10 *anthracis* or a variant thereof which can produce a protective immune response, or (b) a polypeptide comprising at least one protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response as described above, culturing the transformed  
 15 host and recovering the polypeptide therefrom, provided that where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 35%.

20

Using these options, high yields of product can be obtained using a favoured expression host.

A table showing codons and the frequency with which they appear  
 25 in the genomes of *Escherichia coli* and *Bacillus anthracis* is shown in Figure 1. It is clear that guanidine and cytosine appear much more frequently in *E.coli* than *B. anthracis*. Analysis of the codon usage content reveals the following:

Species	1 <sup>st</sup> letter of Codon GC	2nd letter of Codon GC	3rd letter of Codon GC	Total GC content
<i>E. coli</i>	58.50%	40.70%	54.90%	51.37%
<i>B. anthracis</i>	44.51%	31.07%	25.20%	33.59%

30

Thus it would appear that codons which are favoured by *E. coli* are those which include guanidine or cytosine where possible.

By increasing the percentage of guanidine and cytosine nucleotides in the sequence used to encode the immunogenic protein over that normally found in the wild-type *B. anthracis* gene, the codon usage will be such that expression in *E. coli* is improved.

Suitably the percentage of guanidine and cytosine residues within the coding nucleic acid used in the invention, at least where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, is in excess of 40%, preferably in excess of 45% and most preferably from 50-52%.

High levels of expression of protective domains can be achieved, with using the wild-type *B. anthracis* sequence encoding these units. However, the yields may be improved further by increasing the GC% of the nucleic acid as described above.

In a particular embodiment, the method involves the expression of PA of *B. anthracis*.

Further according to the present invention, there is provided a recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

As before, suitably the percentage of guanidine and cytosine residues within the coding nucleic acid is in excess of 40%, preferably in excess of 45% and most preferably from 50-52%.

Suitably, the nucleic acid used to transform the *E. coli* cells of the invention is a synthetic gene. In particular, the



nucleic acid is of SEQ ID NO 1 as shown in Figure 2 or a modified form thereof.

The expression "modified form" refers to other nucleic acid sequences which encode PA or fragments or variants thereof which produce a protective immune response but which utilise some different codons, provided the requirement for the percentage GC content in accordance with the invention is met. Suitable modified forms will be at least 80% similar, preferably 90% similar and most preferably at least 95% similar to SEQ ID NO 1. In particular, the nucleic acid comprises SEQ ID NO 1.

In an alternative embodiment, the invention provides a recombinant *Escherichia coli* cell which has been transformed with a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

Preferably, the nucleic acid encodes domain 1 or domain 4 of *B. anthracis*.

Further according to the invention there is provided a method of producing immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising culturing a cell as described above and recovering the desired polypeptide from the culture. Such methods are well known in the art.

In yet a further aspect, the invention provides an *E. coli* transformation vector comprising a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

A still further aspect of the invention comprises an *E. coli* transformation vector comprising a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective  
5 immune response.

Suitable vectors for use in the transformation of *E. coli* are well known in the art. For example, the T7 expression system provides good expression levels. However a particularly  
10 preferred vector comprises pAG163 obtainable from Avecia (UK).

A nucleic acid of SEQ ID NO 1 or a variant thereof which encodes PA and which has at 35%, preferably at least 40%, more preferably at least 45% and most preferably from 50-52% GC  
15 content form a further aspect of the invention.

If desired, PA of the variants, or domains can be expressed as a fusion to another protein, for example a protein which provides a different immunity, a protein which will assist in  
20 purification of the product or a highly expressed protein (e.g. thioredoxin, GST) to ensure good initiation of translation.

Optionally, additional systems will be added such as T7 lysozyme to the expression system, to improve the repression of the  
25 system, although, in the case of the invention, the problems associated with cell toxicity have not been noted.

Any suitable *E. coli* strain can be employed in the process of the invention. Strains which are deficient in a number of  
30 proteases (e.g.  $\text{lon}^-$ ,  $\text{ompT}^-$ ) are available, which would be expected to minimise proteolysis. However, the applicants have found that there is no need to use such strains to achieve good yields of product and that other known strains such as K12 produce surprisingly high product yields.

Fermentation of the strain is generally carried out under conventional conditions as would be understood in the art. For example, fermentations can be carried out as batch cultures, preferably in large shake flasks, using a complex medium  
5 containing antibiotics for plasmid maintenance and with addition of IPTG for induction.

Suitably cultures are harvested and cells stored at -20°C until required for purification.

10

Suitable purification schemes for *E. coli* PA (or variant or domain) expression can be adapted from those used in *B. subtilis* expression. The individual purification steps to be used will depend on the physical characteristics of recombinant PA.  
15 Typically an ion exchange chromatography separation is carried out under conditions which allow greatest differential binding to the column followed by collection of fractions from a shallow gradient. In some cases, a single chromatographic step may be sufficient to obtain product of the desired specification.

20

Fractions can be analysed for the presence of the product using SDS PAGE or Western blotting as required.

As illustrated hereinafter, the successful cloning and  
25 expression of a panel of fusion proteins representing intact or partial domains of rPA has been achieved. The immunogenicity and protective efficacy of these fusion proteins against STI spore challenge has been assessed in the A/J mouse model.

30 All the rPA domain proteins were immunogenic in A/J mice and conferred at least partial protection against challenge compared to the GST control immunised mice. The carrier protein, GST attached to the N-terminus of the domain proteins, did not impair the immunogenicity of the fusion proteins either in vivo,  
35 shown by the antibody response stimulated in immunised animals, or in vitro as the fusion proteins could be detected with anti-

rPA antisera after Western blotting, indicating that the GST tag did not interfere with rPA epitope recognition. Immunisation with the larger fusion proteins produced the highest titres. In particular, mice immunised with the full length GST 1-4 fusion protein produced a mean serum anti-rPA concentration approximately eight times that of the rPA immunised group (Figure 5). Immunisation of mice with rPA domains 1-4 with the GST cleaved off, produced titres of approximately one half those produced by immunisation with the fusion protein. Why this fusion protein should be much more immunogenic is unclear. It is possible that the increased size of this protein may have an adjuvantising effect on the immune effector cells. It did not stimulate this response to the same extent in the other fusion proteins and any adjuvantising effect of the GST tag did not enhance protection against challenge as the cleaved proteins were similarly protective to their fusion protein counterparts.

Despite having good anti-rPA titres, some breakthrough in protection at the lower challenge level of  $10^2$ MLD's, occurred in the groups immunised with GST1, cleaved 1, GST1b-2, GST1b-3 and GST1-3 and immunisation with these proteins did not prolong the survival time of those mice that did succumb to challenge, compared with the GST control immunised mice. This suggests that the immune response had not been appropriately primed by these proteins to achieve full resistance to the infection. As has been shown in other studies in mice and guinea pigs (Little S.F. et al. 1986. Infect. Immun. 52: 509-512, Turnbull P.C.B., et al., 1986. Infect. Immun. 52: 356-363) there is no precise correlation between antibody titre to PA and protection against challenge. However a certain threshold of antibody is required for protection (Cohen S et. al., 2000 Infect. Immun. 68: 4549-4558), suggesting that cell mediated components of the immune response are also required to be stimulated for protection (Williamson 1989).

GST1, GST1b-2 and GST1-2 were the least stable fusion proteins produced, as shown by SDS-Page and Western blotting results, possibly due to the proteins being more susceptible to degradation in the absence of domain 3, and this instability may  
5 have resulted in the loss of protective epitopes.

The structural conformation of the proteins may also be important for stimulating a protective immune response. The removal of Domain 1a from the fusion proteins gave both reduced  
10 antibody titres and less protection against challenge, when compared to their intact counterparts GST1-2 and GST1-3. Similarly, mice immunised with GST 1 alone were partially protected against challenge, but when combined with domain 2, as the GST1-2 fusion protein, full protection was seen at the  $10^2$   
15 MLD challenge level. However the immune response stimulated by immunisation with the GST1-2 fusion protein was insufficient to provide full protection against the higher  $10^3$  MLD's challenge level, which again could be due to the loss of protective epitopes due to degradation of the protein.

20 All groups immunised with truncates containing domain 4, including GST 4 alone, cleaved 4 alone and a mixture of two individually expressed domains, GST 1 and GST 4 were fully protected against challenge with  $10^3$  MLDs of STI spores (Table  
25 1). Brossier et al showed a decrease in protection in mice immunised with a mutated strain of *B.anthraxis* that expressed PA without domain 4 (Brossier F., et al. 2000. Infect. Immun. 68: 1781-1786) and this was confirmed in this study, where immunisation with GST 1-3 resulted in breakthrough in protection  
30 despite good antibody titres. These data indicate that domain 4 is the immunodominant sub-unit of PA. Domain 4 represents the 139 amino acids of the carboxy terminus of the PA polypeptide. It contains the host cell receptor binding region (Little S.F. et al., 1996 Microbiology 142: 707-715), identified as being in  
35 and near a small loop located between amino acid residues 679-693 (Varughese M., et al. 1999 Infect. Immun. 67:1860-1865).

Therefore it is essential for host cell intoxication as it has been demonstrated that forms of PA expressed containing mutations (Varughese 1999 supra.) or deletions (Brossier 1999 supra.) in the region of domain 4 are non-toxic. The crystal  
5 structure of PA shows domain 4, and in particular a 19 amino acid loop of the domain (703-722), to be more exposed than the other three domains which are closely associated with each other (Petosa 1997 supra.). This structural arrangement may make  
10 domain 4 the most prominent epitope for recognition by immune effector cells, and therefore fusion proteins containing domain 4 would elicit the most protective immune response.

This investigation has further elucidated the role of PA in the stimulation of a protective immune response demonstrating that  
15 protection against anthrax infection can be attributed to individual domains of PA.

The invention will now be particularly described by way of example, with reference to the accompanying drawings in which:  
20

Figure 1 is a Table of codon frequencies found within *E. coli* and *B. anthracis*;

Figure 2 shows the sequence of a nucleic acid according to the  
25 invention, which encodes PA of *B. subtilis*, as published by Welkos et al supra; and

Figure 3 shows SEQ ID NOs 3-14, which are amino acid and DNA sequences used to encode various domains or combinations of  
30 domains of PA as detailed hereinafter;

Figure 4 shows SEQ ID NOs 15-16 which are the amino acid and DNA sequences of domain 4 of PA respectively; and

35 Figure 5 is a table showing anti-rPA IgG concentration, 37 days post primary immunisation, from A/J mice immunised

intramuscularly on days 1 and 28 with 10µg of fusion protein included PA fragment; results shown are mean  $\pm$  sem of samples taken from 5 mice per treatment group.

5 Example 1

Investigation into expression in E.coli

rPA expression plasmid pAG163::rPA has been modified to substitute Km<sup>R</sup> marker for original Tc<sup>R</sup> gene. This plasmid has been transformed into expression host *E. coli* BLR (DE3) and  
10 expression level and solubility assessed. This strain is deficient in the intracellular protease La (Lon gene product) and the outer membrane protease OmpT.

Expression studies did not however show any improvement in the  
15 accumulation of soluble protein in this strain compared to Lon+ K12 host strains (i.e. accumulation is prevented due to excessive proteolysis). It was concluded that any intracellular proteolysis of rPA was not due to the action of La protease.

20 Example 2

Fermentation analysis

Further analysis of the fermentation that was done using the K12 strain UT5600 (DE3) pAG163::rPA.

25 It was found that the rPA in this culture was divided between the soluble and insoluble fractions (estimated 350mg/L insoluble, 650mg/L full length soluble). The conditions used (37°C, 1mM IPTG for induction) had not yielded any detectable soluble rPA in shake flask cultures and given the results  
30 described in Example 1 above, the presence of a large amount of soluble rPA is surprising. Nevertheless it appears that manipulation of the fermentation, induction and point of harvest may allow stable accumulation of rPA in *E. coli* K12 expression strains.

Example 3

A sample of rPA was produced from material initially isolated as insoluble inclusion bodies from the UT5600 (DE3) pAG163::rPA fermentation. Inclusion bodies were washed twice with 25mM  
5 Tris-HCl pH8 and once with same buffer +2M urea. They were then solubilized in buffer +8M urea and debris pelleted. Urea was removed by dilution into 25mM Tris-HCl pH8 and static incubation overnight at 4°C. Diluted sample was applied to Q sepharose column and protein eluted with NaCl gradient. Fractions  
10 containing highest purity rPA were pooled, aliquoted and frozen at -70°C. Testing of this sample using 4-12% MES-SDS NuPAGE gel against a known standard indicated that it is high purity and low in endotoxin contamination.

15 Example 4Further Characterisation of the Product

N terminal sequencing of the product showed that the N-terminal sequence consisted of

20 MEVKQENRLL (SEQ ID NO 2)

This confirmed that the product was as expected with initiator methionine left on.

The material was found to react in Western blot; MALDI -MS on  
25 the sample indicated a mass of approx 82 700 (compared to expected mass of 82 915). Given the high molecular mass and distance from mass standard used (66KDa), this is considered an indication that material does not have significant truncation but does not rule out microheterogeneity within the sample.

30

Example 5Testing of Individual domains of PA

Individual domains of PA were produced as recombinant proteins in *E.coli* as fusion proteins with the carrier protein  
35 glutathione-s-transferase (GST), using the Pharmacia pGEX-6P-3 expression system. The sequences of the various domains and



the DNA sequence used to encode them are attached herewith as Figure 3. The respective amino acid and DNA sequences are provided in Table 2 below.

- 5 These fusion proteins were used to immunise A/J mice (Harlan Olac) intra-muscularly with 10µg of the respective fusion protein adsorbed to 20% v/v alhydrogel in a total volume of 100µl.
- 10 Animals were immunised on two occasions and their development of protective immunity was determined by challenge with spores of B.anthraxis (STI strain) at the indicated dose levels. The table below shows survivors at 14 days post-challenge.

15 **Challenge level in spores/mouse**

Domains	Amino acid SEQ ID NO	DNA SEQ ID NO	5x10 <sup>4</sup>	9x10 <sup>4</sup>	9x10 <sup>5</sup>	1x10 <sup>6</sup>	5x10 <sup>6</sup>
GST-1	3	4	4/4	3/5			
GST-1+2	5	6	4/4; 5/5	4/5; 5/5			
GST-1b+2	7	8	2/5	1/5			
GST-1b+2+3	9	10	2/5	3/5			
GST-1+2+3	11	12	Nd	4/5	3/5		
GST-1+2+3+4	13	14	Nd	5/5	5/5		
1+2+3+4	13	14	Nd	Nd		5/5	5/5

- The data shows that a combination of all 4 domains of PA, whether presented as a fusion protein with GST or not, were protective up to a high challenge level. Removal of domain 4, leaving 1+2+3, resulted in breakthrough at the highest challenge level tested, 9x10<sup>5</sup>. Domains 1+2 were as protective as a combination of domains 1+2+3 at 9x10<sup>4</sup> spores. However, removal of domain 1a to leave a GST fusion with domains 1b+2, resulted in breakthrough in protection at the highest challenge level
- 20

tested ( $9 \times 10^4$ ) which was only slightly improved by adding domain 3.

The data indicates that the protective immunity induced by PA  
5 can be attributed to individual domains (intact domain 1 and domain 4) or to combinations of domains taken as permutations from all 4 domains.

The amino acid sequence and a DNA coding sequence for domain 4  
10 is shown in Figure 4 as SEQ ID NOs 15 and 16 respectively.

#### Example 6

##### Further Testing of domains as vaccines

DNA encoding the PA domains, amino acids 1-259, 168-488, 1-488,  
15 168-596, 1-596, 260-735, 489-735, 597-735 and 1-735 (truncates GST1, GST1b-2, GST1-2, GST1b-3, GST1-3, GST2-4, GST3-4, GST4 and GST1-4 respectively) were PCR amplified from *B. anthracis* Sterne DNA and cloned in to the *XhoI/BamHI* sites of the expression vector pGEX-6-P3 (Amersham-Pharmacia) downstream and in frame of  
20 the *lac* promoter. Proteins produced using this system were expressed as fusion proteins with an N-terminal glutathione-s-transferase protein (GST). Recombinant plasmid DNA harbouring the DNA encoding the PA domains was then transformed in to *E. coli* BL21 for protein expression studies.

25 *E. coli* BL21 harbouring recombinant pGEX-6-P3 plasmids were cultured in L-broth containing 50µg/ml ampicillin, 30µg/ml chloramphenicol and 1% w/v glucose. Cultures were incubated with shaking ( $170 \text{ rev min}^{-1}$ ) at 30°C to an  $A_{600\text{nm}}$  0.4, prior to  
30 induction with 0.5mM IPTG. Cultures were incubated for a further 4 hours, followed by harvesting by centrifugation at 10 000 rpm for 15 minutes.

Initial extraction of the PA truncates-fusion proteins indicated  
35 that they were produced as inclusion bodies. Cell pellets were resuspended in phosphate buffered saline (PBS) and sonicated

4x20 seconds in an iced water bath. The suspension was centrifuged at 15 000 rpm for 15 minutes and cell pellets were then urea extracted, by suspension in 8M urea with stirring at room temperature for 1 hour. The suspension was centrifuged for  
5 15 minutes at 15000 rpm and the supernatant dialysed against 100mM Tris pH 8 containing 400mM L-arginine and 0.1mM EDTA, prior to dialysis into PBS.

The successful refolding of the PA truncate-fusion proteins  
10 allowed them to be purified on a glutathione Sepharose CL-4B affinity column. All extracts (with the exception of truncate GST1b-2, amino acid residues 168-487) were applied to a 15 ml glutathione Sepharose CL-4B column (Amersham-Parmacia), previously equilibrated with PBS and incubated, with rolling,  
15 overnight at 4°C. The column was washed with PBS and the fusion protein eluted with 50mM Tris pH7, containing 150mM NaCl, 1mM EDTA and 20mM reduced glutathione. Fractions containing the PA truncates, identified by SDS-PAGE analysis, were pooled and dialysed against PBS. Protein concentration was determined  
20 using BCA (Perbio).

However truncate GST1b-2 could not be eluted from the glutathione sepharose CL-4B affinity column using reduced glutathione and was therefore purified using ion exchange  
25 chromatography. Specifically, truncate GST1b-2 was dialysed against 20mM Tris pH8, prior to loading onto a HiTrap Q column (Amersham-Parmacia), equilibrated with the same buffer. Fusion protein was eluted with an increasing NaCl gradient of 0-1M in 20mM Tris pH8. Fractions containing the GST-protein were  
30 pooled, concentrated and loaded onto a HiLoad 26/60 Superdex 200 gel filtration column (Amersham-Parmacia), previously equilibrated with PBS. Fractions containing fusion protein were pooled and the protein concentration determined by BCA (Perbio). Yields were between 1 and 43mg per litre of culture.

The molecular weight of the fragments and their recognition by antibodies to PA was confirmed using SDS PAGE and Western Blotting. Analysis of the rPA truncates by SDS Page and Western blotting showed protein bands of the expected sizes. Some degradation in all of the rPA truncates investigated was apparent showing similarity with recombinant PA expressed in *B. subtilis*. The rPA truncates GST1, GST1b-2 and GST1-2 were particularly susceptible to degradation in the absence of domain 3. This has similarly been reported for rPA constructs containing mutations in domain 3, that could not be purified from *B. anthracis* culture supernatants (Brossier 1999), indicating that domain 3 may stabilise domains 1 and 2.

Female, specific pathogen free A/J mice (Harlan UK) were used in this study as these are a consistent model for anthrax infection (Welkos 1986). Mice were age matched and seven weeks of age at the start of the study.

A/J mice were immunised on days 1 and 28 of the study with 10µg of fusion protein adsorbed to 20% of 1.3% v/v Alhydrogel (HCl Biosector, Denmark) in a total volume of 100µl of PBS. Groups immunised with rPA from *B. subtilis* (Miller 1998), with recombinant GST control protein, or fusion proteins encoding domains 1, 4 and 1-4 which had the GST tag removed, were also included. Immunising doses were administered intramuscularly into two sites on the hind legs. Mice were blood sampled 37 days post primary immunisation for serum antibody analysis by enzyme linked immunosorbant assay (ELISA).

Microtitre plates (Immulon 2, Dynex Technologies) were coated, overnight at 4°C with 5µg/ml rPA, expressed from *B. subtilis* (Miller1998), in PBS except for two rows per plate which were coated with 5µg/ml anti-mouse Fab (Sigma, Poole, Dorset). Plates were washed with PBS containing 1% v/v Tween 20 (PBS-T) and blocked with 5% w/v skimmed milk powder in PBS (blotto) for 2 hours at 37°C. Serum, double-diluted in 1% blotto, was added

to the rPA coated wells and was assayed in duplicate together with murine IgG standard (Sigma) added to the anti-fab coated wells and incubated overnight at 4<sup>0</sup> C. After washing, horse-radish peroxidase conjugated goat anti-mouse IgG (Southern  
5 Biotechnology Associates Inc.), diluted 1 in 2000 in PBS, was added to all wells, and incubated for 1 hour at 37<sup>0</sup> C. Plates were washed again before addition of the substrate 2,2'-Azinobis (3-ethylbenzthiazoline-sulfonic acid) (1.09mM ABTS, Sigma). After 20 minutes incubation at room temperature, the absorbance  
10 of the wells at 414nm was measured (Titertek Multiscan, ICN Flow). Standard curves were calculated using Tittersoft version 3.1c software. Titres were presented as µg IgG per ml serum and group means  $\pm$  standard error of the mean (sem) were calculated. The results are shown in Figure 5.

15

All the rPA truncates produced were immunogenic and stimulated mean serum anti-rPA IgG concentrations in the A/J mice ranging from 6µg per ml, for the GST1b-2 truncate immunised group, to 1488µg per ml, in the GST 1-4 truncate immunised group (Figure  
20 5). The GST control immunised mice had no detectable antibodies to rPA.

Mice were challenged with B.anthraxis STI spores on day 70 of the immunisation regimen. Sufficient STI spores for the  
25 challenge were removed from stock, washed in sterile distilled water and resuspended in PBS to a concentration of  $1 \times 10^7$  and  $1 \times 10^6$  spores per ml. Mice were challenged intraperitoneally with 0.1ml volumes containing  $1 \times 10^6$  and  $1 \times 10^5$  spores per mouse, respectively, and were monitored for 14 day post challenge to  
30 determine their protected status. Humane end-points were strictly observed so that any animal displaying a collection of clinical signs which together indicated it had a lethal infection, was culled. The numbers of immunised mice which survived 14 days post challenge are shown in Table 3.

Table 3

Domain	Challenge Level MLDs survivors/no. challenged (%)	
	10 <sup>2</sup> MLDs	10 <sup>3</sup> MLDs
GST 1	3/5 (60)	1/5 (20)
GST 1b-2	1/5 (20)	nd
GST 1-2	5/5 (100)	3/5 (60)
GST 1b-3	3/5 (60)	nd
GST 1-3	4/5 (80)	nd
GST 1-4	nd	5/5 (100)
GST 2-4	nd	5/5 (100)
GST 3-4	nd	5/5 (100)
GST 4	5/5 (100)	5/5 (100)
GST 1+ GST 4	nd	5/5 (100)
Cleaved 1	1/5 (20)	2/5
Cleaved 4	5/5 (100)	5/5
Cleaved 1-4	nd	5/5
rPA	nd	4/4 (100)
control	0/5 (0)	0/5 (0)

- 5 1 MLD = aprox.  $1 \times 10^3$  STI spores  
nd = not done

The groups challenged with 10<sup>3</sup> MLD's of STI spores were all fully protected except for the GST1, GST1-2 and cleaved 1 immunised groups in which there was some breakthrough in protection, and the control group immunised with GST only, which all succumbed to infection with a mean time to death (MTTD) of 2.4 ± 0.2 days. At the lower challenge level of 10<sup>2</sup> MLD's the GST1-2, GST4 and cleaved 4 - immunised groups were all fully protected, but there was some breakthrough in protection in the other groups. The mice that died in these groups had a MTTD of 4.5 ± 0.2 days which was not significantly different from the GST control immunised group which all died with a MTTD of 4 ± 0.4 days.

## Claims

1. An immunogenic reagent which produces an immune response which is protective against *Bacillus anthracis*, said reagent  
5 comprising one or more polypeptides which together represent up to three domains of the full length Protective Antigen (PA) of *B. anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof.  
10
2. An immunogenic reagent according to claim 1 which comprises the sequence of domain 1 and/or domain 4 of wild-type PA.
- 15 3. An immunogenic reagent according to claim 1 or claim 2 which comprises domain 4 of the PA of *B. anthracis*.
4. An immunogenic reagent according to any one of the preceding claims which comprises a combination of domains 1 and  
20 4 or protective regions thereof.
5. An immunogenic reagent according to claim 4 wherein said domains are present in the form of a fusion polypeptide.
- 25 6. An immunogenic reagent according to claim 5 which comprises domain 1 fused to domain 2 of the PA sequence.
7. An immunogenic reagent according to claim 6 which is fused to domain 3 of the PA sequence.  
30
8. An immunogenic reagent according to claim 4 which comprises a mixture of a polypeptides, one of which comprises domain 1 and one of which comprises domain 4 of the PA sequence.

9. An immunogenic reagent according to any one of the preceding claims wherein a polypeptide is fused to a further polypeptide.

5 10. An immunogenic reagent according to claim 9 wherein said further peptide is glutathione-S-transferase (GST).

11. A nucleic acid which encodes a polypeptide of an immunogenic reagent according to any one of the preceding  
10 claims.

12. An expression vector comprising a nucleic acid according to claim 11.

15 13. A cell transformed with a vector according to claim 12.

14. A method for producing an immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising transforming an *E. coli* host  
20 with a nucleic acid which encodes either (a) the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, or (b) a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response,  
25 culturing the transformed host and recovering the polypeptide therefrom, provided that where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* a variant thereof which can produce a protective immune response, the percentage of guanidine and cytosine residues within the said nucleic acid is  
30 in excess of 35%.

15. A method according to claim 14 wherein the said nucleic acid encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune  
35 response.



16. A method according to claim 15 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 45%.

5 17. A method according to claim 16 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is from 50-52%.

10 18. A method according to claim 14 wherein the said nucleic acid encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

15 19. A method according to claim 18 wherein the domain is domain 1 and/or domain 4 of PA of *B. anthracis*.

20 20. A recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

25 21. A recombinant *Escherischia coli* cell according to claim 20 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 45%.

30 22. A recombinant *Escherischia coli* cell according to claim 21 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is from 50%-52%.

23. A recombinant *E. coli* cell according to claim 20 wherein said nucleic acid is of SEQ ID NO 1 as shown in Figure 2 or a modified form thereof.

24. A recombinant *E. coli* cell according to claim 23 wherein said nucleic acid is of SEQ ID NO 1.

25. A recombinant *Escherischia coli* cell which has been  
5 transformed with a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

26. A recombinant cell according to claim 25 wherein the  
10 nucleic acid encodes domain 1 or domain 4 of PA of *B. anthracis*.

27. A method of producing a polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising culturing a cell according to any one of  
15 claims 20 to 26 and recovering the protective polypeptide from the culture.

28. An *E. coli* transformation vector comprising a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis*  
20 or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

29. An *E. coli* transformation vector comprising a nucleic acid  
25 which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

30. A nucleic acid of SEQ ID NO 1 or a modified form thereof  
30 which encodes PA or a variant thereof which produces a protective immune response and which has at least 35% GC content.

31. A nucleic acid according to claim 30 which is at least 90%  
35 identical to SEQ ID NO 1.

32. A nucleic acid according to claim 31 which comprises SEQ ID NO 1.

34. A method of preventing or treating infection by *B. anthracis*, said method comprising administering to a mammal in need thereof, a sufficient amount of an immunogenic reagent according to any one of claims 1 to 10.

35. The use of an immunogenic reagent according to any one of claims 1 to 10 in the preparation of a medicament for the prophylaxis or treatment of *B. anthracis* infection.

***Escherichia coli* [gbbet]: 14457 CDS's (4541860 codons)****Fields: [triplet] [frequency: per thousand] ([number])**

UUU 22.0(100128)	UCU 9.3( 42367)	UAU 16.7( 75774)	UGU 5.2( 23461)
UUC 16.5( 74885)	UCC 8.9( 40365)	UAC 12.3( 55847)	UGC 6.3( 28747)
UUA 13.8( 62823)	UCA 7.9( 35837)	UAA 2.0( 9006)	UGA 1.0( 4428)
UUG 13.3( 60322)	UCG 8.7( 39546)	UAG 0.3( 1172)	UGG 14.5( 65630)
CUU 11.3( 51442)	CCU 7.2( 32678)	CAU 12.7( 57585)	CGU 20.7( 93997)
CUC 10.6( 48147)	CCC 5.4( 24383)	CAC 9.6( 43743)	CGC 21.1( 96053)
CUA 4.0( 18067)	CCA 8.5( 38663)	CAA 14.8( 67129)	CGA 3.7( 16607)
CUG 50.9(231373)	CCG 22.3(101467)	CAG 28.8(130898)	CGG 5.7( 25751)
AUU 29.9(135873)	ACU 9.5( 43256)	AAU 18.7( 84846)	AGU 9.1( 41544)
AUC 24.6(111878)	ACC 22.7(103121)	AAC 21.6( 98018)	AGC 15.6( 70867)
AUA 5.3( 24233)	ACA 7.9( 35995)	AAA 34.4(156169)	AGA 2.7( 12345)
AUG 27.2(123604)	ACG 14.0( 63696)	AAG 11.4( 51685)	AGG 1.6( 7423)
GUU 19.1( 86572)	GCU 16.2( 73677)	GAU 32.3(146794)	GGU 25.1(114185)
GUC 14.8( 67356)	GCC 25.0(113412)	GAC 19.3( 87759)	GGC 28.6(130043)
GUA 11.2( 51020)	GCA 20.6( 93390)	GAA 39.5(179460)	GGA 8.6( 39036)
GUG 25.5(115687)	GCG 32.2(146264)	GAG 18.5( 83804)	GGG 11.1( 50527)

Coding GC 51.37% 1<sup>st</sup> letter GC 58.50% 2<sup>nd</sup> letter GC 40.70% 3<sup>rd</sup> letter GC 54.90%***Bacillus anthracis* [gbbet]: 180 CDS's (52031 codons)****Fields: [triplet] [frequency: per thousand] ([number])**

UUU 33.5( 1745)	UCU 17.3( 902)	UAU 34.4( 1792)	UGU 6.1( 319)
UUC 10.2( 530)	UCC 5.3( 275)	UAC 9.4( 490)	UGC 2.1( 107)
UUA 44.2( 2301)	UCA 14.0( 730)	UAA 2.3( 119)	UGA 0.5( 24)
UUG 11.3( 589)	UCG 3.6( 188)	UAG 0.7( 37)	UGG 9.8( 511)
CUU 14.7( 763)	CCU 10.1( 525)	CAU 16.8( 873)	CGU 10.9( 567)
CUC 3.7( 195)	CCC 2.7( 141)	CAC 4.6( 239)	CGC 2.6( 137)
CUA 13.2( 686)	CCA 14.9( 773)	CAA 33.7( 1752)	CGA 6.8( 353)
CUG 4.7( 242)	CCG 4.6( 237)	CAG 10.4( 542)	CGG 1.8( 95)
AUU 44.6( 2322)	ACU 14.6( 761)	AAU 44.6( 2321)	AGU 16.5( 861)
AUC 11.8( 616)	ACC 5.2( 269)	AAC 13.7( 711)	AGC 5.1( 266)
AUA 24.9( 1295)	ACA 25.9( 1350)	AAA 69.5( 3614)	AGA 13.8( 720)
AUG 23.8( 1240)	ACG 8.1( 419)	AAG 23.5( 1223)	AGG 4.3( 226)
GUU 19.9( 1036)	GCU 17.9( 930)	GAU 39.7( 2068)	GGU 17.3( 900)
GUC 5.2( 268)	GCC 4.7( 244)	GAC 8.8( 456)	GGC 5.4( 279)
GUA 26.8( 1395)	GCA 22.6( 1178)	GAA 55.7( 2897)	GGA 20.2( 1049)
GUG 9.7( 507)	GCG 7.1( 368)	GAG 19.3( 1003)	GGG 8.9( 461)

Coding GC 33.59% 1<sup>st</sup> letter GC 44.51% 2<sup>nd</sup> letter GC 31.07% 3<sup>rd</sup> letter GC 25.20%

Figure 1

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1  AAGCTTCATA TGGAAGTAAA GCAAGAGAAC CGTCTGCTGA ACGAATCTGA ATCCAGCTCT
61 CAGGGCCTGC TTGGTTACTA TTTCTCTGAC CTGAACTTCC AAGCACCGAT GGTGTGAACC
121 AGCTCTACCA CTGGCGATCT GTCCATCCCG TCTAGTGAAC TTGAGAACAT TCCAAGCGAG
181 AACCAGTATT TCCAGTCTGC AATCTGGTCC GGTTTTATCA AAGTCAAGAA ATCTGATGAA
241 TACACGTTTG CCACCTCTGC TGATAACCAC GTAACCATGT GGGTTGACGA TCAGGAAGTG
301 ATCAACAAAG CATCCAACTC CAACAAAATT CGTCTGGAAG AAGGCCGTCT GTATCAGATC
361 AAGATTCACT ACCAACGCGA GAACCCGACT GAAAAAGGCC TGGACTTTAA ACTGTATTGG
421 ACTGATTCTC AGAACAGAA AGAAGTGATC AGCTCTGACA ATCTGCAACT GCCGGAATTG
481 AAACAGAAAA GCTCCAACTC TCGTAAGAAA CGTTCCACCA GCGCTGGCCC GACCGTACCA
541 GATCGCGACA ACGATGGTAT TCCGGACTCT CTGGAAGTTG AAGGCTACAC GGTGTGATGA
601 AAGAACAAC GTACCTTCCT TAGTCCGTGG ATCTCCAATA TTCACGAGAA GAAAGGTCTG
661 ACCAAATACA AATCCAGTCC GGAAAAATGG TCCACTGCAT CTGATCCGTA CTCTGACTTT
721 GAGAAAGTGA CCGGTCGTAT CGACAAGAAC GTCTCTCCGG AAGCACGCCA TCCACTGGTT
781 GCTGCGTATC CGATCGTACA TGTTGACATG GAAAACATCA TTTTGTCCAA GAACGAAGAC
841 CAGTCCACTC AGAACACTGA CTCTGAAACT CGTACCATCT CCAAGAACAC TCACACGTCT
901 CGTACTCACA CCAGTGAAGT ACATGGTAAC GCTGAAGTAC ACGCCTCTTT CTTTGACATC
961 GCGGCTCTG TTAGCGCTGG CTTCTCCAAC TCTAATTCTT CTACTGTTGC CATTGATCAC
1021 TCTCTGAGTC TGGCTGGCGA ACGTACCTGG GCAGAGACCA TGGGTCTTAA CACTGCTGAT
1081 ACCGCGCGTC TGAATGCTAA CATTGCTACT GTCAACACTG GTACGGCACC GATCTACAAC
1141 TACTGCCAA CCACCAGCCT GGTCTGGGT AAGAACCAGA CTCTTGCGAC CATCAAAGCC
1201 AAAGAGAACC AACTGTCTCA GATTCTGGCA CCGAATAACT ACTATCCTTC CAAGAACCTG
1261 GCTCCGATCG CACTGAACGC ACAGGATGAC TTCTCTTCCA CTCCGATCAC CATGAACCTAC
1321 AACCAGTTCC TGGAACCTGA GAAGACCAA CAGCTGCGTC TTGACACTGA CCAAGTGTAC
1381 GGTAACATCG CGACCTACAA CTTTGAGAAC GGTCGCGTCC GCGTTGACAC AGGCTCTAAT
1441 TGGTCTGAAG TACTGCCTCA GATTCAGGAA ACCACCGCTC GTATCATCTT CAACGGTAAA
1501 GACCTGAACC TGGTTGAACG TCGTATTGCT GCTGTGAACC CGTCTGATCC ATTAGAGACC
1561 ACCAAACCGG ATATGACTCT GAAAGAAGCC CTGAAGATCG CCTTTGGCTT CAACGAGCCG
1621 AACGGTAATC TTCAGTACCA AGGTAAAGAC ATCACTGAAT TTGACTTCAA CTTTGATCAG
1681 CAGACCTCTC AGAATATCAA GAACCAACTG GCTGAGCTGA ACGCGACCAA TATCTATACG
1741 GTACTCGACA AGATCAAAC GAACGCGAAA ATGAACATTC TGATTGCGCA CAAACGTTTC
1801 CACTACGATC GTAATAACAT CGCTGTTGGC GCTGATGAAT CTGTTGTGAA AGAAGCGCAT
1861 CGCGAAGTCA TCAACTCCAG CACCGAAGGC CTGCTTCTGA ACATCGACAA AGACATTGCT
1921 AAGATCCTGT CTGGTTACAT TGTTGAGATC GAAGACACCG AAGGCCTGAA AGAAGTGATC
1981 AATGATCGTT ACGACATGCT GAACATCAGC TCTCTGCGTC AAGATGGTAA GACGTTTATT
2041 GACTTCAAGA AATACAACGA CAACTTCCG CTGTATATCT CTAATCCGAA CTACAAAGTG
2101 AACGTTTACG CTGTTACCAA AGAGAACACC ATCATCAATC CATCTGAGAA CGGCGATACC
2161 TCTACCAACG GTATCAAGAA GATTCTGATC TTCTCCAAGA AAGGTTACGA GATCGGTTAA
2221 TAGGATCC

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(SEQ ID No 1)

Figure 2

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1 EVKQENRLLN ESESSSQGLL GYFSDLNFO APMVVTSSST GDLSIPSSSEL ENIPSENQYF
61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTKGL DFKLYWTD SQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAA

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(Seq ID No 3)

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1 gaagttaaacc aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta
61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcgaaaaa ccaatatttt
181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaatata aattcaatat
361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
421 aataaaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaaatct
481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
601 acttttcttt caccatggat ttctaataat catgaaaaga aaggattaac caaatataaa
661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaagggttaca
721 ggacgggattg ataagaatgt atcaccagag gcaagacacc cccttgtggc agct

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(Seq ID No 4)

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1 EVKQENRLLN ESESSSQGLL GYFSDLNFO APMVVTSSST GDLSIPSSSEL ENIPSENQYF
61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTKGL DFKLYWTD SQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRHTT
301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL
361 NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
481 LPQIQET

```

(SEQ ID No 5)

Figure 3

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1  gaagttaaac  aggagaaccg  gttatttaa  gaatcagaat  caagttccca  ggggttacta
61  ggatactatt  ttagtgattt  gaattttcaa  gcacccatgg  tggttacttc  ttctactaca
121  ggggatttat  ctattcctag  ttctgagtta  gaaaatattc  catcggaaaa  ccaatatattt
181  caatctgcta  tttggtcagg  atttatcaaa  gttaagaaga  gtgatgaata  tacatttgct
241  acttccgctg  ataatcatgt  aacaatgtgg  gtagatgacc  aagaagtgat  taataaagct
301  tctaattcta  acaaaatcag  attagaaaaa  ggaagattat  atcaaatata  aaticaatat
361  caacgagaaa  atcctactga  aaaaggattg  gatttcaagt  tgtactggac  cgattotcaa
421  aataaaaaag  aagtgatttc  tagtgataac  ttacaactgc  cagaattaaa  acaaaaatct
481  tcgaactcaa  gaaaaaagcg  aagtacaagt  gctggaccta  cggttccaga  ccgtgacaat
541  gatggaatcc  ctgattcatt  agaggtagaa  ggatatacgg  ttgatgtcaa  aaataaaaga
601  acttttcttt  caccatggat  ttctaataat  catgaaaaga  aaggattaac  caaatataaa
661  tcatctcctg  aaaaatggag  cacggcttct  gatccgtaca  gtgatttcga  aaaggttaca
721  ggacggattg  ataagaatgt  atcaccagag  gcaagacacc  cccttggtgc  agcttatccg
781  attgtacatg  tagatatgga  gaatattatt  ctctcaaaaa  atgaggatca  atccacacag
841  aatactgata  gtgaaacgag  aacaataagt  aaaaatactt  ctacaagtag  gacacatact
901  agtgaagtac  atggaaatgc  agaagtgcac  gcgtcgttct  ttgatattgg  tgggagtgtg
961  tctgcaggat  ttagtaattc  gaattcaagt  acggtcgcaa  ttgatcattc  actatctcta
1021  gcaggggaaa  gaacttgggc  tgaaacaatg  ggtttaaata  ccgctgatac  agcaagatta
1081  aatgccaata  ttagatatgt  aaatactggg  acggctccaa  tctacaacgt  gttaccaacg
1141  acttoggtag  tgtaggaaa  aaatacaaca  ctgcgcacaa  ttaaagctaa  ggaaaaccaa
1201  ttaagtcaaa  tacttgacc  taataattat  tatccttcta  aaaacttggc  gccaatcgca
1261  ttaaatgcac  aagacgattt  cagttctact  ccaattacaa  tgaattacaa  tcaatttctt
1321  gagttagaaa  aaacgaaaca  attaagatta  gatacggatc  aagtatatgg  gaatatagca
1381  acatacaatt  ttgaaaatgg  aagagtgagg  gtggatacag  gtcgaactg  gagtgaagtg
1441  ttaccgcaaa  ttcaagaaac  a

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(SEQ ID No 6)

```

1  SAGPTVPDRD  NDGIPDSLEV  EGYTVDVKNK  RTFLSPWISN  IHEKKGLTKY  KSSPEKWSTA
61  SDPYSDFEKV  TGRIDKNVSP  EARHPLVAAY  PIVHVDMENI  ILSKNEDQST  QNTDSETRTI
121  SKNTSTSRTH  TSEVHGNAEV  HASFFDIGGS  VSAGFSNSNS  STVAIDHSLS  LAGERTWAET
181  MGLNTADTAR  LNANIRYVNT  GTAPIYNVLP  TTSLVLGKNQ  TLATIKAKEN  QLSQILAPNN
241  YYP SKNLAPI  ALNAQDDFSS  TPITMNYNQF  LELEKTKQLR  LDTDQVYGN  ATYNFENG RV
301  RVD TGSNWSE  VLPQIQET

```

(SEQ ID No 7)

Figure 3 Cont.

```

1 agtgctggac ctacggttcc agaccgtgac aatgatggaa tccctgattc attagaggta
61 gaaggatata cggttgatgt caaaaataaa agaacttttc tticaccatg gatttctaata
121 attcatgaaa agaaaggatt aaccaaatat aaatcatctc ctgaaaaaatg gagcacggct
181 tctgatccgt acagtgattt cgaaaagggt acaggacgga ttgataagaa tgtatcacca
241 gaggcaagac acccccttgt ggcagcttat ccgattgtac atgtagatat ggagaatatt
301 attctctcaa aaaatgagga tcaatccaca cagaatactg atagtgaac gagaacaata
361 agtaaaaata cttctacaag taggacacat actagtgaag tacatggaaa tgcagaagtg
421 catgcgtcgt tctttgatat tgggtgggagt gtatctgcag gatttagtaa ttcgaattca
481 agtacgggtcg caattgatca ttcactatct ctagcagggg aaagaacttg ggctgaaaca
541 atggggtttaa ataccgctga tacagcaaga ttaaagtcca atattagata tgtaataact
601 gggacggctc caatctacaa cgtgttacca acgacttcgt tagtgttagg aaaaaatcaa
661 aactcgcgga caattaaagc taaggaaaac caattaagtc aaatacttgc acctaataat
721 tattatcctt ctaaaaactt ggcgccaatc gcattaaatg cacaagacga tttcagttct
781 actccaatta caatgaatta caatcaattt cttgagttag aaaaaacgaa acaattaaga
841 ttagatacgg atcaagtata tgggaatata gcaacataca attttgaaaa tggaagagtg
901 aggggtggata caggctcgaa ctggagtga gttgtaccgc aaattcaaga aaca

```

(SEQ ID No 8)

```

1 SAGPTVPDRD NDGIPDSLEV EGYTVDVKNK RTFLSPWISN IHEKKGLTKY KSSPEKWSTA
61 SDPYSDFEKV TGRIDKNVSP EARHPLVAAY PIVHVDMENI ILSKNEDQST QNTDSETRTI
121 SKNTSTSRTH TSEVHGNAEV HASFFDIGGS VSAGFSNSNS STVAIDHSLs LAGERTWAET
181 MGLNTADTAR LNANIRYVNT GTAPIYNVLP TTSLVLGKNQ TLATIKAKEN QLSQILAPNN
241 YYPskNLAPI ALNAQDDFSS TPITMNYNQF LELEKTKQLR LDTDQVYGNi ATYNFENGRV
301 RVDtGSNWSE VLPQIQETTA RIIFNGKDLN LVERRIAAVN PSDPLETTKP DMTLKEALKI
361 AFGFNEPNGN LQYQgKDITE FDFNFDQOTS QNIKNQLAEL NATNIYTVLD KIKLNAKMNI
421 LIRDKR

```

(SEQ ID No 9)

Figure 3. Cont.



```

1 agtgctggac ctacggttcc agaccgtgac aatgatggaa tccctgattc attagaggta
61 gaaggatata cggttgatgt caaaaataaa agaacttttc ttccaccatg gatttctaata
121 attcatgaaa agaaaggatt aaccaaatat aaatcatctc ctgaaaaatg gagcacggct
181 tctgatccgt acagtgattt cgaaaagggt acaggacgga ttgataagaa tgtatcacca
241 gaggaagac acccccttgt ggcagcttat ccgattgtac atgtagatat ggagaatatt
301 attctctcaa aaaatgagga tcaatccaca cagaatactg atagtgaac gagaacaata
361 agtaaaaata cttctacaag taggacacat actagtgaag tacatggaaa tgcagaagtg
421 catgcgtcgt tctttgatat tgggtggagt gtatctgcag gatttagtaa ttcgaattca
481 agtacggtcg caattgatca ttcactatct ctagcagggg aaagaacttg ggctgaaaca
541 atgggtttaa ataccgctga tacagcaaga ttaaattgcca atattagata tgtaaatact
601 gggacggctc caatctacaa cgtgttacca acgacttcgt tagtgtagg aaaaaatcaa
661 acactcgcca caattaaagc taaggaaaac caattaagtc aaatacttgc acctaatat
721 tattatcctt ctaaaaactt ggcgccaatc gcattaaatg cacaagacga tttcagttct
781 actocaatta caatgaatta caatcaattt cttgagttag aaaaaacgaa acaattaaga
841 ttagatacgg atcaagtata tgggaatata gcaacatata attttgaaaa tggaagagtg
901 aggggtgata caggctcgaa ctggagtga gttgtaccgc aaattcaaga acaactgca
961 cgtatcattt ttaattgaaa agattttaat ctggtagaaa ggcggatagc ggcggttaat
1021 cctagtgatc cattagaaac gactaaaccg gatatgacat taaaagaagc ccttaaaata
1081 gcatttggat ttaacgaacc gaatggaaac ttacaatatc aagggaaga cataaccgaa
1141 tttgatttta atttcgatca acaaacatct caaaatatca agaatcagtt agcggaatta
1201 aacgcaacta acatatatac tgtattagat aaatcaaata taaatgcaaa aatgaatatt
1261 ttaataagag ataaacgt

```

(SEQ ID No 10)

```

1 EVKQENRLLN ESESSSQGLL GYFSDLNFO APMVVSSTT GDLSIPSSEL ENIPSENQYF
61 QSAIWSGFIK VKKSDEYTF A TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTKGL DFKLYWTD SQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTV DVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRHTT
301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIHSLSL AGERTWAETM GLNTADTARL
361 NANIRYVNTG TAPIYNVLP TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
481 LPQIQET TAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL
541 QYQGDITEF DFNFDQOTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKR

```

(SEQ ID No 11)

Figure 3 Cont.

```

1 gaagttaaac aggagaacog gttattaaat gaatcagaat caagttccca ggggttacta
61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt
181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaataaaa aattcaatat
361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgatttctca
421 aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct
481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga cctgacaat
541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
601 acttttcttt caccatggat ttctaataat catgaaaaga aaggattaac caaatataaa
661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcca aaaggttaca
721 ggacggattg ataagaatgt atcaccagag gcaagacacc cccttggtgc agcttatccg
781 attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag
841 aatactgata gtgaaacgag aacaataagt aaaaatactt ctacaagtag gacacatact
901 agtgaagtac atggaatgc agaagtgc atgcgtcgttct ttgatattgg tgggagtgt
961 tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta
1021 gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata cgcgtgatac agcaagatta
1081 aatgccaaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg
1141 acttctgttag tgttaggaaa aaatcaaaaca ctgcgcgaca ttaaagctaa ggaaaaccaa
1201 ttaagtcaaa tacttgcacc taataattat tacccttcta aaaacttggc gccaatcgca
1261 ttaaattgcac aagacgattt cagtttctact ccaattacaa tgaattacaa tcaatttctt
1321 gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca
1381 acatacaatt ttgaaaatgg aagagtggag gtggatacag gctcgaactg gagtgaagt
1441 ttaccgcaaa ttcaagaaac aactgcacgt atcattttta atggaaaaga tttaaatctg
1501 gtagaaaggc ggatagcggc gggttaatcct agtgatccat tagaaacgac taaaccggat
1561 atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaaactta
1621 caatatcaag ggaagacat aacogaattt gattttaatt togatcaaca aacatctcaa
1681 aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa
1741 atcaaattaa atgcaaaaat gaatatttta ataagagata aacgt

```

(SEQ ID No 12)

```

1 EVKQENRLLN ESESSSQGLL GYFSDLNFO APMVVTSSST GDLSIPSEL ENIPSENQYF
61 QSAIWSGFIK VKKSDEYTF TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTKGL DFKLYWTD SQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSQTRTIS KNTSTSRTHT
301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL
361 NANIRYVNTG TAPIYNVLP TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
481 LPQIQET TAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL
541 QYQKGDITEF DFNFDQOTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
601 NNIAGVADES VVKEAHREVI NSSTEGLLN IDKDIRKILS GYIVEIEDTE GLKEVINDRY
661 DMLNISSLRQ DGKTFIDFKK YNDKLPYIIS NPNYKVNVA VTKENTIINP SENGDTSTNG
721 IKKILIFSCK GYEIG

```

(SEQ ID No 13)

Figure 3 Cont.

```

1  gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta
61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatattt
181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat
361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
421 aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct
481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
601 acttttcttt caccatggat ttctaataat catgaaaaga aaggattaac caaatataaa
661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca
721 ggacggattg ataagaatgt atcaccagag gcaagacacc cccttggtgc agcttatccg
781 attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag
841 aatactgata gtgaaacgag aacaataagt aaaaataact ctacaagtag gacacatact
901 agtgaagtac atggaaatgc agaagtgc atgcgtcttct ttgatattgg tgggagtgtg
961 tctgcaggat ttagtaattc gaattcaagt acggtcgc aa ttgatcattc actatctcta
1021 gcaggggaaa gaacttgggc tgaacaatg ggtttaaata ccgctgatac agcaagatta
1081 aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg
1141 acttcgttag tgtaggaaa aaatcaaa ctcgcgacaa ttaaagctaa ggaaaaccaa
1201 ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca
1261 ttaaattgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt
1321 gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca
1381 acatacaatt ttgaaaatgg aagagtggag gtggatacag gctcgaactg gagtgaagtg
1441 ttaccgcaaa ttcaagaaac aactgcacgt atcattttta atggaaaaga tttaaatctg
1501 gtagaaaggc ggatagcggc ggttaatcct agtgatccat tagaaacgac taaaccggat
1561 atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaactta
1621 caatatcaag ggaaagacat aaccgaattt gattttaatt tcgatcaaca aacatctcaa
1681 aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa
1741 atcaaattaa atgcaaaaat gaatatttta ataagagata aacgttttca ttatgataga
1801 aataacatag cagttggggc ggatgagtca gtagttaagg aggctcatag agaagtaatt
1861 aattcgtcaa cagagggatt attgttaa attgataagg atataagaaa aatattatca
1921 gggttatattg tagaaattga agatactgaa gggcttaaag aagttataaa tgacagatat
1981 gatatgttga atatttctag ttacggcaa gatggaaaaa catttataga ttttaaaaaa
2041 tataatgata aattaccgtt atatataagt aatcccaatt ataaggtaaa tgtatatgct
2101 gttactaaag aaacactat tattaatcct agtgagaatg gggatactag taccaacggg
2161 atcaagaaaa ttttaatctt ttctaaaaaa ggctatgaga taggataa

```

(SEQ ID No 14)

Figure 3 Cont.

1 FHYDRNNIAV GADESVVKEA HREVINSSTE GLLLNIDKDI RKILSGYIVE IEDTEGLKEV  
61 INDRYDMLNI SSLRQDGKTF IDFKKYNDKL PLYISNPNYK VNVYAVTKEN TIINPSENGD  
121 TSTNGIKKIL IFSKKGYEIG

(SEQ ID No 15)

1 tttcattatg atagaaataa catagcagtt ggggcggatg agtcagtagt taaggaggct  
61 catagagaag taattaattc gtcaacagag ggattattgt taaatattga taaggatata  
121 agaaaaatat tatcaggtta tattgtagaa attgaagata ctgaagggct taaagaagtt  
181 ataaatgaca gatatgatat gttgaatatt tctagtttac ggcaagatgg aaaaacattt  
241 atagatttta aaaaatataa tgataaatta ccgttatata taagtaatcc caattataag  
301 gtaaatgtat atgctgttac taaagaaaac actattatta atcctagtga gaatggggat  
361 actagtacca acgggatcaa gaaaatttta atcttttcta aaaaaggcta tgagatagga  
421 taa

(SEQ ID No 16)

Figure 4

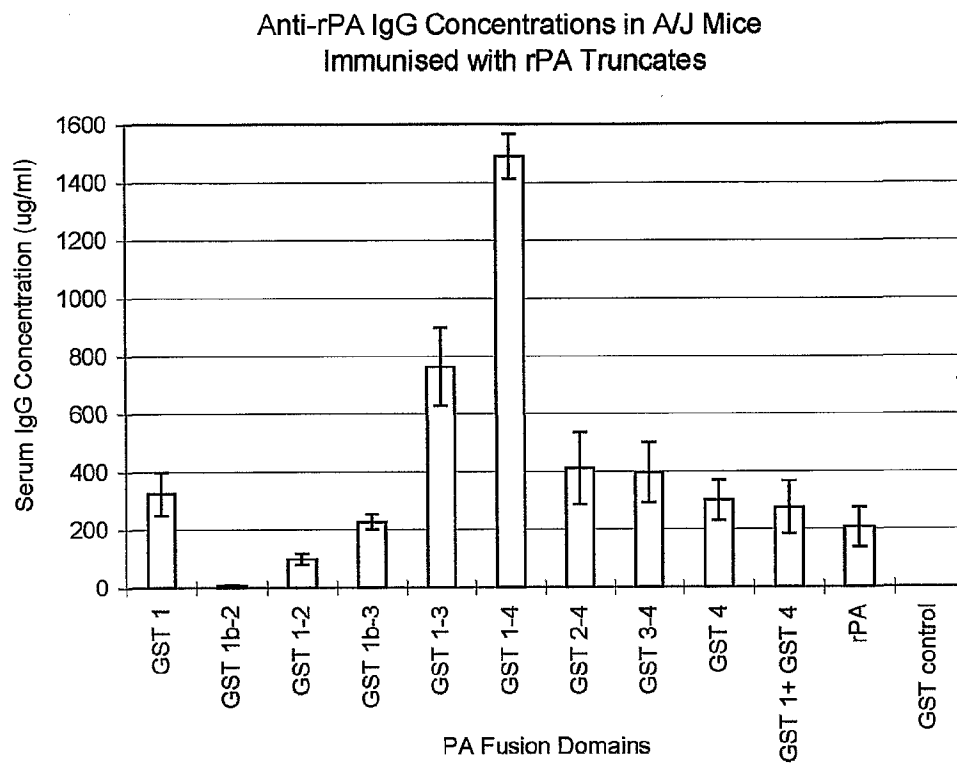


Figure 5

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/03065

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/70 C07K14/32 A61K39/07 C12N15/31 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BROSSIER FABIEN ET AL: "Role of toxin functional domains in anthrax pathogenesis."</p> <p>INFECTION AND IMMUNITY, vol. 68, no. 4, April 2000 (2000-04), pages 1781-1786, XP002183267</p> <p>ISSN: 0019-9567</p> <p>figure 1</p> <p>page 1785, left-hand column, last paragraph -right-hand column</p> <p>-----</p> <p>-/--</p>	<p>1-23,</p> <p>25-31,</p> <p>34,35</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

16 November 2001

Date of mailing of the international search report

30/11/2001

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/03065

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 5 677 274 A (NICHOLS PETER J ET AL) 14 October 1997 (1997-10-14) column 12, last paragraph - column 13, paragraph 2 ---	14-32
A	WELKOS S L ET AL: "SEQUENCE AND ANALYSIS OF THE DNA ENCODING PROTECTIVE ANTIGEN OF BACILLUS-ANTHRACIS" GENE (AMSTERDAM), vol. 69, no. 2, 1988, pages 287-300, XP002183269 ISSN: 0378-1119 figure 2 -----	1-35

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International Application No  
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